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Suramin Blocks Hepatitis C Binding to Human Hepatoma Cells In Vitro

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It was demonstrated recently that the binding of dengue virus to its target cell receptor could be effectively blocked by both heparin and by the polysulphonate pharmaceutical, Suramin [Chen et al. (1997) Nature Medicine 3:866–871]. Because both dengue and hepatitis C virus (HCV) belong to the *Flaviviridae* and because the HCV envelope is predicted to possess a heparin-binding motif, we tested heparin, Suramin, and a number of other polyanionic compounds for their ability to block HCV binding in vitro. The compounds, at concentrations ranging from 0.5 to 5,000 μg/ml, were tested using the human hepatoma cell line HepG2 cultured under conditions designed to enhance hepatocyte differentiation. Cells were harvested at 2 weeks postinoculation and HCV-RNA was quantified by means of a chemiluminescent reverse transcription polymerase chain reaction (PCR) assay. Suramin was found to be capable of blocking HCV binding in this system at a concentration similar to that reported to be effective against dengue virus. Removal of the viral envelope by treatment with chloroform also prevented HCV infection. Neither chondroitin sulphate nor the Suramin analogue CPD14 were able to block HCV under these conditions. ***J. Med. Virol. 57: 238–242, 1999.***

KEY WORDS: HCV; tissue culture; HepG2; polyanions; sulphated polysaccharides

INTRODUCTION

Chronic infection with hepatitis C virus (HCV) constitutes a major public health problem in many parts of the world and available antiviral therapies remain inadequate [Schalm and Brouwer, 1997]. Unfortunately, the development and screening of antiviral compounds has been hampered severely by the lack of a reliable in vitro system for HCV propagation. In an attempt to address this problem, a culture method based on the human hepatoma cell line HepG2 [Aden et al., 1979] was developed.

Many previous attempts to infect human hepatoma cell lines with HCV have been unsuccessful [Shimizu et al., 1992; Iacovacci et al., 1993], possibly due to inadequate expression of differentiated hepatocyte-specific functions by such lines under standard culture conditions. We therefore elected to culture the HepG2 line using a medium designed empirically to enhance hepatocyte differentiation [Moshage and Yap, 1992; Goss, 1993]. In addition, we used an insulin/dexamethasone exposure step, which has been shown to facilitate the infection of hepatoma cells with hepatitis B virus in vitro [Bchini et al., 1990].

The results are described of experiments designed to test the ability of certain polyanionic compounds, including sulphated polysaccharides and Suramin, to prevent HCV infection of HepG2 cells. Sulphated polysaccharides were selected for evaluation following the discovery by Chen et al. [1997] that the cellular receptor for dengue virus is a highly sulphated form of heparan sulphate. Chen et al. [1997] demonstrated that the binding of dengue virus to its target cell receptor could be blocked effectively by both heparin and by the polysulphonate pharmaceutical, Suramin. Because both dengue and HCV belong to the family of *Flaviviridae* and because the amino acid sequence of the HCV envelope protein is predicted theoretically to possess a heparin-binding motif [Chen et al., 1997], both heparin and Suramin were examined for their ability to reduce the yield of HCV in the HepG2 culture system.

METHODS

Cell Culture Prior to Inoculation

The human hepatoma cell line HepG2 [Aden et al., 1979] was obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium supplemented with Glutamax-1 (Gibco), 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (2.5 μg/ml). Cells were grown as monolayers in 75-cm^2 culture flasks coated with a 2% solution of collagen (Vitrogen

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TABLE I. Composition of Tissue Culture Media*

Component	Concentration	Reference[a]
G_{basal} medium		
Dimethyl sulfoxide (DMSO)	1.7%	Gripon et al., 1988
Forskolin[b]	5×10^{-6} M	Goss, 1993
Phorbol myristate acetate[b]	1.6×10^{-7} M	Meyers et al., 1992
Retinol acetate[b]	5.6 IU/ml	Grunt et al., 1991
Butyric acid (Na salt)	0.5 mM	Moshage and Yap, 1992
Niacinamide	10 mM	Moshage and Yap, 1992
Hexadimethrine bromide (Polybrene)	2×10^{-6}g/ml	Coelen et al., 1983
Sodium selenite	2.9×10^{-8} M	
Lipids, cholesterol-rich (Sigma # L-4646)	0.25%	Gherardi et al., 1992
β-Estradiol	3.7×10^{-8} M	Gherardi et al., 1992
3′,5-Triiodo-L-thyronine, Na salt	1×10^{-9} M	Gherardi et al., 1992
Ultroser®-G serum substitute (Gibco)	2%	Moshage and Yap, 1992
Penicillin	100 U/ml	
Streptomycin	100 μg/ml	
Fungizone® (Gibco)	2.5 μg/ml	
G_{pulse} medium		
As G_{basal} medium, but also contains:		
Dexamethasone[b]	1.3×10^{-4} M	Bchini et al., 1990
Insulin	2.7×10^{-5} M	Bchini et al., 1990
G_{maint} medium		
As G_{pulse} medium, but with:		
Dexamethasone[b]	1.3×10^{-6} M	Bchini et al., 1990
Insulin	2.7×10^{-6} M	Bchini et al., 1990

*All media based on RPMI 1640 + Glutamax-1. After preparation, the pH of the medium was adjusted where necessary by adding 1 M NaOH dropwise until the indicator colour returned to orange (pH 7.2). Anti-human interferon (1000 IU/ml, Sigma) was present in all media from inoculation until terminal harvest at 2 weeks postinoculation.

[a]References relate to use of these medium components to enhance cellular differentiation or viral entry and infection in other culture systems.

[b]These stock reagents were prepared in DMSO. The final 1.7% concentration of DMSO takes this into account.

100, Imperial Labs Ltd) at 37°C in a 5% CO_2 atmosphere.

Inoculation Procedure

A confluent 75-cm^2 flask of HepG2 cells was harvested by trypsinisation and the cells washed and resuspended at 5×10^5 cells/ml in G_{basal} medium (Table I). Five hundred microliters of cell suspension was added per well of a 24-well Natrix-coated [Moshage and Yap, 1992] tissue culture plate (Becton Dickinson). Duplicate wells were used for each experiment. The cells were allowed to settle for 24 hr at 37°C and then inoculated with HCV containing serum "JR" (HCV-RNA titre 2.5×10^6 genomes/ml, genotype 1a, anti-HCV negative by Abbott 2nd generation enzyme-linked immunosorbent assay [ELISA]) diluted to 1% in G_{basal} medium. After 24 hr at 37°C, the inoculum was removed and replaced by 500 μl of G_{pulse} medium (Table I). Following a 5-hr incubation at 37°C, the cells were washed twice with 2 ml of RPMI-1640, then 500 μl of G_{maint} medium (Table I) were added to each well. Cells were cultured for 2 weeks at 37°C in a humidified 5% CO_2 atmosphere with a G_{maint} medium change after 7 days.

Quantitative Polymerase Chain Reaction (PCR) for HCV-RNA

At 2 weeks postinoculation, cell monolayers were washed twice (2 ml per wash), harvested by trypsinisation, and resuspended in 1.5 ml RPMI 1640 + 10% FBS. Cells were then pelleted by centrifugation and resuspended in 210 μl phosphate-buffered saline. Two hundred microliters of the cell suspension was used for RNA extraction by the acid guanidinium method of Chomczynski and Sacchi [1987] and the remaining 10 μl used for cell count and viability assay. HCV-RNA titre was measured by means of a chemiluminescence-based quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay as described previously [Whitby and Garson, 1997]. The assay has a lower detection limit of approximately 80 HCV genomes per million cells. PCR contamination prevention measures based on the principles of Kwok and Higuchi [1989] were used throughout and at least three negative control samples were included in each experimental run.

Prevention of HCV Infection

In some experiments, the lipid envelope of HCV was removed by treating the "JR" inoculum with chloroform; 250 μl of chloroform were added to 500 μl of "JR" serum (diluted 1:10 in G_{basal} medium) and mixed by inversion once per minute for 20 min at room temperature. The chloroform was removed following 4°C centrifugation at 15,000 rpm for 5 min.

Alternatively, immediately prior to inoculation, 1% "JR" serum was incubated in G_{basal} medium for 1 hr at 37°C with serial dilutions of various polyanionic compounds (Sigma, St. Louis, MO) including heparin, chondroitin sulphate A, dextran sulphate (MW 5×10^5), Suramin, and the Suramin analogue CPD14

[Braddock et al., 1994]. These agents remained present throughout the 24-hr inoculation period and were then removed along with the inoculum. Control incubations (1 hr at 37°C) of the inoculum with RPMI in place of the test compounds were also undertaken.

Cell Viability Assays and Cell Cycle Analysis

Cell counts and viability were assessed routinely by Trypan blue exclusion when the cells were harvested at 2 weeks postinoculation. In one experiment, cell cycle analysis was carried out by propidium iodide staining and flow cytometry [Elstein et al., 1995] on uninoculated HepG2 cells following 2 weeks of culture in G_{maint} medium. In addition, the compounds were tested for toxicity on HepG2 cells cultured in G_{basal} medium by means of an acid phosphatase-based cell viability assay, as described previously [Yang et al., 1996].

RESULTS

Effects of G Medium on HepG2 Cells

Unlike HepG2 cells, which form relatively chaotic monolayers when grown under standard conditions, cells cultured in the presence of a cocktail of differentiation inducing agents (G medium, Table I) form a highly regular pavement-like epithelium with well-defined cellular borders. In addition, G medium leads to a dramatic reduction in the rate of cell division so that splitting the cultures every third day is unnecessary. Cell viability was maintained at approximately 75% for several weeks and cell cycle analysis by propidium iodide staining confirmed that most of the cells were arrested in G_1, with only 2% in S phase.

HCV Infection of HepG2 Cell Line Cultured Under Differentiating Conditions

Preliminary experiments with HepG2 cells cultured under standard conditions (RPMI 1640 + FBS or synthetic serum substitute) demonstrated that they could not be infected with HCV. However, when cultured under differentiating conditions in G medium, HCV-RNA became detectable in HepG2 cell pellets for at least 6 weeks postinoculation (p.i.). The quantity of HCV-RNA in the cell pellets increased gradually from around day 7 postinoculation, reaching a plateau of approximately 3×10^3 to 3×10^4 HCV genomes per million cells by day 14. Infection was found to be dependent on both the particular inoculum and the particular cell line used; several other liver-derived, epithelial, and lymphoid cell lines were found to be uninfectable or yielded much lower levels of HCV. Control wells without cells or wells inoculated with normal human serum in place of "JR" serum gave consistently negative results.

Effect of Chloroform Pretreatment of Inoculum

Removal of the lipid envelope of HCV by treatment of the inoculum with chloroform completely prevented infection of the HepG2 cell line (Fig. 1). Control experiments demonstrated that the titre and stability of HCV-RNA in the inoculum was not affected by the chloroform treatment.

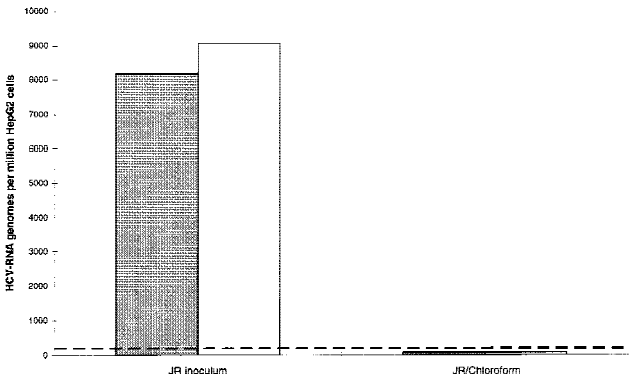

Fig. 1. HepG2 cells inoculated with untreated 'JR' serum or with chloroform treated 'JR' serum as described under Methods. Bars with dark shading and light shading show HCV-RNA yield in HepG2 cells harvested at 1 and 2 weeks p.i. respectively. The horizontal dashed line represents the lower detection limit of the PCR assay.

Effect of Sulphated Polysaccharides and Suramin

Heparin (4–400 μg/ml), dextran sulphate (20–2,000 μg/ml), and Suramin (50–5,000 μg/ml) all appeared to be capable of preventing HCV infection in this system. However, the significance of the results obtained with heparin and dextran sulphate is uncertain because residual traces of both of these compounds were found to co-purify with the viral RNA and inhibited the RT-PCR assay used for HCV detection. In contrast, experiments with Suramin did not demonstrate any such inhibition of the RT-PCR assay. A representative dose response curve for the blockade of HCV infection by Suramin is shown in Figure 2. The concentration of Suramin (~100 μg/ml) required to reduce the yield of HCV by 50% had no adverse effect on cell viability as judged by either Trypan blue exclusion or acid phosphatase-based assays. Treatment of the HCV-infected HepG2 culture for 24 hr with 4,000 μg/ml of Suramin during the second week postinoculation had no significant effect on the yield of HCV.

Preincubation of the "JR" serum for 1 hr at 37°C with RPMI in place of Suramin did not impair the ability of the inoculum to infect the HepG2 cell line. Neither chondroitin sulphate (2,000 μg/ml) nor the Suramin analogue, CPD14 (4,000 μg/ml), were able to block HCV infection of HepG2 cells under these conditions.

DISCUSSION

Although early attempts to infect human hepatoma cell lines with HCV were uniformly unsuccessful [Shimizu et al., 1992; Iacovacci et al., 1993], a recent report by Siepp et al. [1997] suggested that infection may be achieved using serum-free medium supplemented with polyethylene glycol, dimethyl sulphoxide, and lovastatin. The yield of HCV-RNA obtained in the present study was similar to that reported by Siepp et al. [1997] (~ 10^4 HCV genomes per million cells) and somewhat higher than that seen in the human embryonic hepatocyte cell line WRL68 (~10^2 HCV genomes

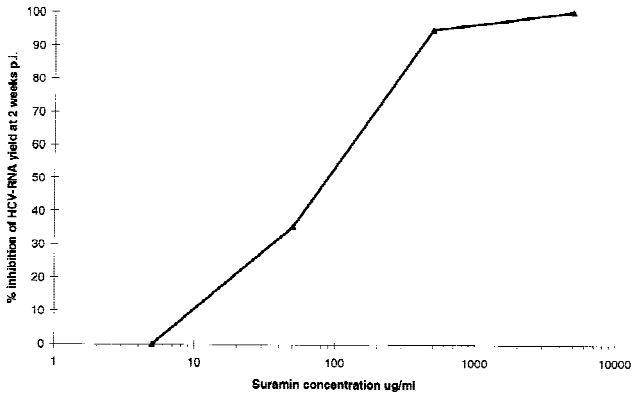

Fig. 2. Typical dose response curve for Suramin showing inhibition of hepatitis C virus (HCV) infection. Suramin was present throughout the inoculation period as described under Methods.

per million cells; Tagawa et al., 1995) or in human fetal liver cell cultures [Iacovacci et al., 1993]. The ability of chloroform treatment of the inoculum to prevent infection in vitro (Fig. 1) is consistent with its effect on enveloped viruses in vivo [Feinstone et al., 1983].

The identification of highly sulphated heparan sulphate as the receptor for dengue virus [Chen et al., 1997] prompted us to test the ability of heparin to block HCV infection in the HepG2 system. Unfortunately, the interpretation of this and of a similar experiment with dextran sulphate was complicated by the fact that both of these substances interfered with the assay used for the detection of HCV. This problem was not altogether unexpected, because heparin is a well-known potent inhibitor of PCR [Holodniy et al., 1991] and dextran sulphate has been shown to inhibit reverse transcriptase [Baba et al., 1988]. Nevertheless, dextran sulphate has been reported recently to block the adsorption of HCV to peripheral blood mononuclear cells [Cribier et al., 1998].

Chen et al. [1997] demonstrated that in addition to heparin, the polysulphonate pharmaceutical, Suramin, was able to block dengue virus infection in vitro. Suramin was therefore tested in the HepG2/HCV system and found to inhibit HCV infection at a similar concentration (Fig. 2) to that reported to be effective against dengue. Unlike the experiments with heparin and dextran sulphate, the interpretation of the Suramin data was not complicated by inhibition of the RT-PCR assay. Although Suramin prevented infection when it was present before and during the inoculation period, the compound had no effect when applied some days after inoculation. This finding is consistent with the hypothesis that Suramin acts at the initial stages of the interaction between the virus and the cell, perhaps by blocking the viral envelope–cell receptor interaction, as suggested in the dengue system [Chen et al., 1997]. The ability of Suramin to prevent HCV infection of HepG2 cells is unlikely to be simply a nonspecific effect of any polyanionic compound, because neither the structurally related Suramin analogue CPD14 nor the sulphated polysaccharide chondroitin sulphate had an anti-HCV effect in this system.

Suramin has been used for over half a century in the treatment of various parasitic infections; during the past few years the compound has been investigated as a potential anti-cancer agent [Braddock et al., 1994]. Suramin also has anti-HIV activity and Yahi et al. [1994] demonstrated that it blocks the binding of the V3 region of the HIV-1 envelope glycoprotein gp 120 to galactosylceramide, an alternative receptor for HIV on human neural and colonic epithelial cells. In addition, Suramin has been shown to inhibit in vitro infection by duck hepatitis B virus, Rous sarcoma virus, and hepatitis delta virus, by blocking virus uptake [Tsiquaye et al., 1986; Petcu et al., 1988]. However, Loke et al. [1987] reported that Suramin was not effective against hepatitis B virus in vivo in humans. Unfortunately, the clinical application of Suramin is restricted significantly by its undesirable side effects. At the concentrations required to block HCV infection in the HepG2 system, clinical toxicity is predicted [Kobayashi et al., 1995]. However, a number of less toxic Suramin analogues have been designed and one of these, CPD14, which in mice is 5- to 10-fold less toxic than Suramin, was tested in the present study. Although CPD14 proved ineffective here, we consider that it may well be worth investigating alternative Suramin analogues in this system.

In conclusion, the HCV culture system described in this study appears to be suitable for the investigation of candidate antiviral compounds, such as Suramin, that are thought to work by blocking viral binding and uptake. However, care must be taken to ensure that any apparent antiviral activity is not simply due to inhibition of the RT-PCR assay used for HCV detection. Efforts are underway to evaluate the system for the screening of antiviral candidates that act at later stages in the virus replication cycle.

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